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CONTRACTING ORGANIZATION: University of Alabama at Birmingham
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ABSTRACT

During the last three years of support of the career development award, we sought to develop and evaluate recombinant adeno-associated virus vectors encoding anti-angiogenic factors. We have completed the work with significant outcome, and also initiated new studies that will allow us to test novel possibilities for breast cancer treatment. The outcome of the present studies strongly indicated that sustained expression of anti-angiogenic factors not only provide significant growth inhibition of localized cancer in mouse model but also exerted tumor-free survival in combination with chemotherapy. We have also identified that the effect of anti-angiogenic therapy with rAAV in combination with chemotherapy, was independent of survivin pathway. Histopathological analysis of major visceral organs and liver enzyme analysis also revealed no toxicity because of combination therapy.

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Principal Investigator: Selvarangan Ponnazhagan, Ph.D.

INTRODUCTION

The mortality associated with breast cancer is primarily due to systemic dissemination of the disease to which conventional therapies such as surgery, radiation therapy and chemotherapy fail to provide long-term cure. Thus, development of novel approaches is important for the treatment of metastatic breast disease. Among the possible targets, the tumor endothelium is more promising since the endothelial cell growth, termed angiogenesis, is a crucial event for tumor growth and metastasis (1-3). Earlier studies using purified anti-angiogenic factors indicated a need for constant administration, which involves complexities in production, and possible side effects. To overcome these limitations, more recently, approaches based on the introduction of anti-angiogenic genes using plasmid DNA, adenoviral, and retroviral vectors are being attempted. However, these vectors have limitations such as inefficient long-term persistence, host immunity and a requirement of active cell division respectively.

Recombinant adeno-associated virus (rAAV) vectors, on the other hand, are unique group of DNA containing viruses, which can transduce both dividing and non-dividing cells. rAAV vectors are less immunogenic and establish long-term persistence in host cells, hence, possess the advantage for sustained long-term expression of the transgenes. Based on these advantages, we hypothesized that long-term gene therapy for breast cancer by AAV-mediated sustained expression of anti-angiogenic factors *in vivo* will be efficacious both as a primary therapy for established tumors and as an adjuvant therapy for the recurrence of radiation- and chemo-resistant tumors. Further, we hypothesized that development of breast cancer-specific rAAV containing anti-angiogenic genes would not only increase targeted-transduction but also minimize the vector dose and associated toxicity, if any. The proposed specific aims of the project are:

1) To determine the anti-angiogenic potential of rAAV encoding angiostatin, endostatin and soluble VEGF receptor in biologically relevant assays.

2) To evaluate the *in vivo* efficacy of rAAV encoding the anti-angiogenic factors in inhibiting breast cancer growth and metastasis in a murine model.

3) To determine the efficacy of anti-angiogenic gene therapy as an adjuvant therapy for recurrence of radiation resistant breast cancer growth and metastasis in a murine model *in vivo*.

BODY

During the last three years of support of the career development award, we sought to develop and evaluate recombinant adeno-associated virus vectors encoding anti-angiogenic factors. We have completed the work with significant outcome, and also initiated new studies that will allow us to test novel possibilities for breast cancer treatment. The outcome of the present studies strongly indicated that sustained expression of anti-angiogenic factors not only provide significant growth inhibition of localized cancer in mouse model but also exerted tumor-free survival in combination with chemotherapy. We have also identified that the effect of anti-angiogenic therapy with rAAV in combination with chemotherapy, was independent of survivin pathway. One of the cellular mechanisms by which breast cancer cells become resistant to chemotherapy is by overexpressing survivin. Survivin is a member of the Inhibitor of Apoptosis Protein (IAP) family of anti-apoptotic proteins, which suppresses cell death by inhibiting caspase family cell death proteases. Most human cancers inappropriately over-express survivin, making it one of the most tumor-specific transcripts detected to date in genome-wide surveys. Survivin has been found to be highly expressed in rapidly dividing cells but undetectable in normal differentiated tissues. Thus, we studied if synergistic effect of rAAV anti-angiogenic therapy and chemotherapy is imparted by overcoming survivin-mediated effects. These studies were first established *in vitro* to demonstrate that SKOV3.ip1 cells that we used in all the *in vivo* experiments increase survivin expression upon treatment with taxol but not following rAAV transduction. Then, explanted tumors from naïve mice or that received taxol, rAAV or taxol plus rAAV were immunohistochemically stained with human survivin antibody. The same slides were also stained with βIII tubulin antibody for colocalization. Results, given in Figure 1, demonstrated that rAAV transduction. Histopathological analysis of major visceral organs and liver enzyme analysis also revealed no toxicity because of combination therapy (Figure 2).

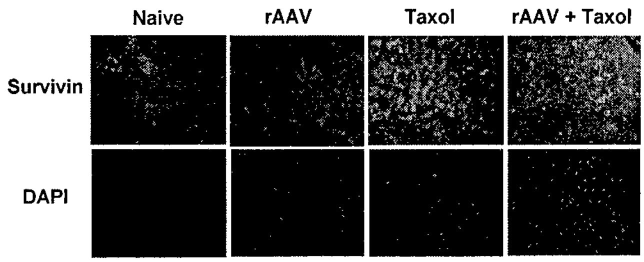

Figure 1: Survivin expression in tumor tissues of naïve, taxol, rAAV and taxol+rAAV treated mice. Cellular survivin were examined by immunohistochemical staining of explanted tumor tissues using anti-human survivin antibody (Santa Cruz Biotechnology, Inc.). DNA was stained with 4'-6-Diamidino-2-phenylindole (DAPI, Molecular Probes Inc). Magnification x200.

Based on our studies so far, we have initiated new line of work to utilize rAAV for targeted delivery of siRNA in breast cancer *in vivo* to down-regulate the expression of transforming growth factor (TGF)-β, known to play key role in breast cancer growth, metastasis and immunosuppression. I plan to develop work in this area and seek extramural funding from various sources including the DoD.

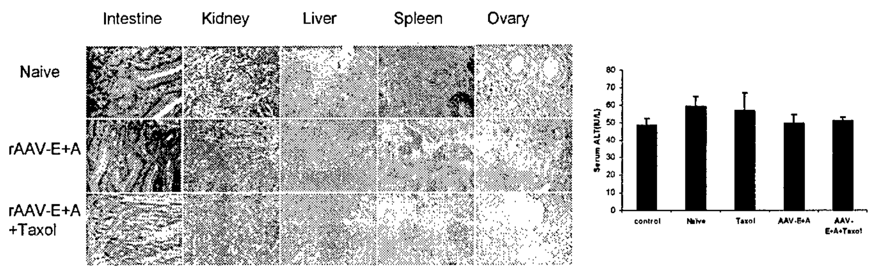

Figure 2: Histopathology and liver enzyme for toxicity analysis. To determine if rAAV-E+A therapy, either alone or in combination with chemotherapy results in long-term hepatic and other organ toxicity, histopathological analysis of major visceral organs was performed by hematoxylin and eosin staining (left). As a measure of hepatoxicity, alanine aminotransferase levels were determined in serum samples by a colorimetric assay (right).

KEY RESEARCH ACCOMPLISHMENTS

1. Established that rAAV-mediated gene therapy with anti-angiogenic factors angiostatin, endostatin provided significant anti-tumor effect in combination with the chemotherapeutic drug taxol independent of survivin pathway.
2. Long-term expression of angiostatin and endostatin and stable systemic production following rAAV-mediated gene transfer does not cause hepatic or organ toxicity.

REPORTABLE OUTCOMES

(Papers published or communicated)

- Mahendra, G., Kumar, S., Mahasreshti, P., Curiel, D.T., Stockardt, C.R., Grizzle, W.E., Alapati, V., Singh, R., Siegal, G.P., Meleth, S., and **Ponnazhagan, S.** Anti-angiogenic cancer gene therapy by adeno-associated virus 2-mediated stable expression of the soluble *FMS*-related tyrosine kinase-1 receptor. *Cancer Gene Ther.* 2005, 12: 26-34.

- Isayeva, T., Ren, C., and **Ponnazhagan, S.** Recombinant adeno-associated virus 2 -mediated anti-angiogenic prevention in a mouse model of intraperitoneal ovarian cancer. *Clin. Cancer Res.* 2005, 11:1342-1347.

(Results presented in conferences)

- Chaudhuri, T.R., Cao, Z., Ponnazhagan, S., Stargel, A., Simhadri, P.L., Zhou, T., LoBuglio, A.F., Buchsbaum, D.J., and Zinn, K. Detection of disseminated breast cancer growth and treatment response using non-invasive bioluminescence imaging (BI). 40th Annual meeting of the American Society of Clinical Oncology, New Orleans, LA, June 2004.
- Isayeva, T., Ren, C., and Ponnazhagan, S. Recombinant adeno-associated virus 2 - mediated anti-angiogenic gene therapy in a mouse model of intraperitoneal ovarian cancer. 7th Annual meeting of the American Society of Gene Therapy, Minneapolis, MN, June 2004.
- Ponnazhagan, S., Mahendra, G., Kumar, S., Shaw, D.R., Stockard, C.R., Grizzle, W.E., and Meleth, S. Adeno-associated virus 2-mediated anti-angiogenic cancer gene therapy: long-term efficacy of a vector encoding angiostatin and endostatin over vectors encoding a single factor. 7th Annual meeting of the American Society of Gene Therapy, Minneapolis, MN, June 2004.
- Isayeve, T., Ren, C., and Ponnazhangan, S. Intraperitoneal transduction of adeno-associated virus 2 expressing angiostatin and endostatin synergistically augments paclitaxel therapy and tumor-free survival in a mouse model of epithelial ovarian cancer, 96th Annual Meeting of the American Association for Cancer Research, April 2005 Anaheim, CA.
- Isayeve, T., Ren, C., and Ponnazhangan, S. Adeno-associated virus mediated anti-angiogenic gene therapy. Department of Defense – Breast Cancer Research Program, 4th Era of Hope Meeting, Philadelphia, PA, June 2005.

CONCLUSIONS

- Recombinant adeno-associated virus vectors encoding angiostatin and endostatin were developed.
- The biological activity of rAAV produced endostatin and angiostatin was determined in *in vitro* assays.

- *In vivo* anti-tumor effects of rAAV encoding endostatin and angiostatin were determined in athymic nude mouse model.
- Combination of chemotherapy with rAAV anti-angiogenic therapy was found to provide significant anti-tumor effects without systemic and organ toxicity.
- The combination therapy was found to be independent of survivin expression.

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2. Folkman , J., Tumor angiogenesis: therapeutic implications. N. Engl. J. Med., 1971,285:1182-6.
3. Folkman, J. Anti-angiogenic gene therapy. Proc. Natl. Acad. Sci. U.S.A. 1998,95:9064-9066.

PERSONNEL RECEIVING PAY FROM THIS GRANT

Selvarangan Ponnazhagan, Ph.D.

APPENDICES

Copies of the following manuscripts enclosed:

- Mahendra, G., Kumar, S., Mahasreshti, P., Curiel, D.T., Stockardt, C.R., Grizzle, W.E., Alapati, V., Singh, R., Siegal, G.P., Meleth, S., and **Ponnazhagan, S.** Anti-angiogenic cancer gene therapy by adeno-associated virus 2-mediated stable expression of the soluble *FMS*-related tyrosine kinase-1 receptor. *Cancer Gene Ther.* 2005, 12: 26-34.

- Isayeva, T., Ren, C., and **Ponnazhagan, S.** Recombinant adeno-associated virus 2 -mediated anti-angiogenic prevention in a mouse model of intraperitoneal ovarian cancer. *Clin. Cancer Res.* 2005, 11:1342-1347.

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Antiangiogenic cancer gene therapy by adeno-associated virus 2-mediated stable expression of the soluble FMS-like tyrosine kinase-1 receptor

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Antiangiogenic gene transfer has the potential to be more efficacious than protein-based therapies or pharmacotherapies for the control of solid tumor growth, invasion and metastasis. For a sustained antiangiogenic effect, a vector capable of long-term expression without vector-associated immunity or toxicity is advantageous. The present study evaluated the potential of a recombinant adeno-associated virus-2 (rAAV) encoding the human soluble FMS-like tyrosine kinase receptor 1 (sFlt-1), which functions by both sequestering vascular endothelial growth factor (VEGF) and forming inactive heterodimers with other membrane-spanning VEGF receptors, *in vitro* and *in vivo*. Results indicated significant growth inhibitory activity of the transgenic factor in a human umbilical vein endothelial cell proliferation assay *in vitro* and protection against the growth of an angiogenesis-dependent human ovarian cancer cell line, SKOV3.ip1, xenograft *in vivo* with increased disease-free survival. Stable expression of the secretory factor and transgene persistence were confirmed by immunohistochemistry and *in situ* hybridization analyses, respectively. Increased therapeutic effects on both the growth index of the implanted tumor cells and tumor-free survival also correlated with an increasing dose of the vector used. These studies indicate that rAAV-mediated sFlt-1 gene therapy may be a feasible approach for inhibiting tumor angiogenesis, particularly as an adjuvant/therapy.
Cancer Gene Therapy (2005) **12**, 26–34. doi:10.1038/sj.cgt.7700754
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***Keywords:* adeno-associated virus; anti-angiogenesis**

A better understanding of the interaction of normal host and tumor cells has provided clues towards developing novel therapies for cancer. Cancer gene therapy targeting non-neoplastic cells has recently shown greater potential against tumor growth and metastasis in preclinical models. One of the promising areas of cancer therapy is targeting the growth of tumor-associated endothelium, which provides anchorage and nourishment for the growth of solid tumors through the process of angiogenesis.[1] It has been clearly established that in the absence of angiogenesis, tumors fail to grow beyond 2–3 mm^3 in size and remain dormant.[1,2] Thus, by providing antiangiogenic signals through sustained systemic expression of the inhibitory factors at therapeutic levels, it may be possible to control the growth of solid tumors. Further, antiangiogenic gene therapy can be combined with conventional therapies such as radiation or chemotherapy or newer therapies including immunotherapy for synergistic effects.[3]

For sustained expression of antiangiogenic factors at a therapeutic level by gene transfer, an important requirement is the use of vectors capable of stable expression without the possibility of elimination of transgene-positive cells by T-cell-mediated cytolysis. Adeno-associated virus (AAV)-based vectors have shown the potential for long-term expression of therapeutic genes without such vector-associated immunity or toxicity.[4–7] Since antiangiogenic gene therapy mandates a sustained expression of the antiangiogenic factors at a therapeutic level, recombinant(r) AAV is an ideal vector to accomplish this.

Soluble FMS-like tyrosine kinase receptor 1 (sFlt-1) is a splice variant of the vascular endothelial growth factor (VEGF) receptor Flt-1, lacking the transmembrane and cytoplasmic domains.[8] VEGF is a potent angiogenic factor, and overexpression of VEGF has been reported to be associated with poor prognosis in many human

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cancers.[9–13] The VEGF antagonistic activity of sFlt-1 is effected both by forming inactive heterodimers with membrane-spanning VEGF receptor and by sequestering VEGF in a dominant-negative manner, thereby inhibiting the downstream VEGF signaling cascade following receptor-mediated internalization.[8] Thus, use of sFlt-1 for downregulating VEGF signaling at two different steps would maximize the process of inhibiting tumor neovascularization and associated tumor growth. The present study demonstrates that stable expression of sFlt-1 following rAAV-mediated gene transfer provides significant protection against the growth of an angiogenesis-dependent human ovarian cancer cell line in a mouse xenograft model suggesting its potential application in antiangiogenic cancer gene therapy in human beings.

Materials and methods

Cells and reagents

The human embryonic kidney cell line, 293, was purchased from the ATCC and maintained in Iscove's modified essential medium supplemented with 10% fetal bovine serum. Human ovarian cancer cell line SKOV3.ip1 was a kind gift of Dr Janet Price (MD Anderson Cancer Center, Houston, TX) and was maintained as before.[14] Primary human umbilical vein endothelial cells (HUVEC) were obtained from Dr Francoise Booyse (The University of Alabama at Birmingham, AL) and maintained as before.[15] Restriction endonucelases and other modifying enzymes were purchased from either New England Biolabs (Beverly, MA) or Promega Corporation (Madison, WI). A mouse monoclonal anti-human VEGF receptor-1 (Flt-1 receptor) antibody, which recognizes the extracellular domain of Flt-1 (represented in the sFlt-1) was purchased from Sigma Chemicals (V4262, St Louis, MO). A rat anti-mouse monoclonal antibody for CD31 (PECAM-1) was purchased from Chemicon International (CBL1337, Temecula, CA). Secondary antibodies and color reagents were purchased from Abcam Inc. (Cambridge, MA) and Amersham (Piscataway, NJ). Purified recombinant human sFlt-1 was purchased from R&D Systems Inc. (Minneapolis, MN).

Construction of recombinant plasmids, production and purification of rAAV

All rAAV plasmids were constructed using pSub201 as the backbone.[16] cDNA encoding human sFlt-1 was cloned from a human placental cDNA library as described.[17] The coding sequences were initially subcloned in a mammalian expression vector pCI (Promega, Madison, WI) under the control of the CMV promoter and a cassette comprising the CMV promoter, sFlt-1 gene and polyadenylation signal sequence was isolated and subcloned in pSub201, replacing the AAV-2 genes. Packaging of rAAV-sFlt-1 was accomplished in an adenovirus-free system as described.[18] Purification of the virions was carried out in a discontinuous iodixanol gradient centrifugation followed by affinity purification in heparin affinity column. The particle titer of the purified virions was determined by quantitative slot blot analysis as described.[19,20]

Western blot analysis

Western blot analysis was performed using conditioned media obtained from rAAV-transduced 293 cells. Briefly, conditioned media collected following mock-transduction (without any vector) or rAAV-sFlt-1 transduction was concentrated five-fold and 20 μl from each aliquot was electrophoretically separated in a 10% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). The gel was transferred to a PVDF membrane and immunodetection of the proteins was performed using a monoclonal antibody directed against the extracellular region of the human sFlt-1. A goat anti-mouse secondary antibody, conjugated to horseradish peroxidase (HRP), was used as a secondary antibody. Detection of the bands was performed using an enhanced chemiluminescent (ECL) system as described.[21]

Endothelial cell proliferation assay

Early passage HUVEC were seeded in gelatin-coated 96-well tissue culture plates at a density of 5×10^3 cells per well and grown in EGM-2 medium containing hydrocortisone, human FGF-β, VEGF, ascorbic acid, heparin, human EGF and 10% fetal bovine serum (Clonetics Corp., San Diego, CA). After 24 hours, the medium was changed and 100 μl of fresh medium containing the same additive plus 20 μl of conditioned medium obtained from 293 cells that were either mock-transduced or transduced with 100 multiplicities of infection (MOI) of rAAV-sFlt-1. As a positive control, purified recombinant human angiostatin (R&D Systems, Minneapolis, MN) was added in a separate well at a concentration of 20 μg/ml. After 72 hours, the cells were fixed in 10% buffered-formalin and stained with 1% crystal violet in 70% ethanol as described[22] to analyze the rate of proliferation.

In vivo *studies*

Female athymic nude mice, 6 weeks old, were purchased from the Fredericks Cancer Institute, NCI, and housed in the animal facility of the University of Alabama at Birmingham (UAB). The animals were maintained following the Guidelines of the Institutional Animal Care and Use Committee (IACUC) after all experimental procedures were approved by both the UAB-IACUC and the Occupational Health and Safety Department. The animals were divided into six groups consisting of six mice per group. A total of 3×10^{11} particles of rAAV encoding either GFP, or sFlt-1, suspended in normal saline, were injected in a volume of 50–100 μl in the quadriceps muscle of the hind limb. Naïve animals did not receive any vector. At 3 weeks after vector administration, each mouse was implanted with 10^7 SKOV3.ip1 cells, subcutaneously. A total of two injections were delivered per mouse, one in each flank. Tumor size was measured twice a week with a digital caliper for two-dimensional longest

axis (L in mm) and shortest axis (W in mm), and tumor volume calculated using the formula: volume in $mm^3 = (L \times W^2)/2$. If the tumor growth exceeded $1800\,mm^3$, the animals were euthanized. The surviving mice were killed by the end of 130 days after tumor cell implantation, and the experiment terminated. At the time of killing either due to tumor burden or termination of the experiment, both the liver and regions of the quadriceps muscle at the site of sham or vector injection were isolated and processed for total DNA isolation, histology, immunohistochemistry and *in situ* hybridization.

Histological analyses of liver tissue

Liver tissues were harvested from naïve or rAAV-sFlt1-treated mice. The tissues were immediately fixed in 10% buffered-formalin (pH 7.0) and embedded in paraffin following standard processing methods. Sections of 5 μm thickness were cut, deparaffinized in xylene and dehydrated in alcohol. The slides were hematoxylin and eosin (H&E) stained and mounted with cover slips. Analysis of the stained sections was carried out by a senior histopathologist by standard bright-field microscopy.[23]

PCR analysis for vector genome

Total DNA was isolated from naïve or rAAV-sFlt1 administered mouse muscle using TRIizol reagent (GIBCO-BRL). PCR amplification was carried out in a 30-cycle reaction using a primer-pair, specific for the amplification of the vector genome. The forward primer consisted sequence of CMV promoter and the reverse primer that of the human sFlt-1. The primer sequences were: forward primer 5′-TAAGCAGAGCTCGTT TAGTGAACCGT-3′ and reverse primer, 5′- TACTCAC CATTTCAGGCAAAGACCAT-3′. The amplified product (548 bp) was electrophoretically separated on 1% agarose gels and the bands visualized by ethidium bromide staining.

Immunohistochemistry

The quadriceps muscle of mice were harvested under anesthesia and fixed immediately in alcoholic-formalin (PenFix; Richard-Allan, Kalamazoo, MI) for 18 hours at room temperature. The tissues were dehydrated in graded alcohol and embedded in paraffin. Sections of 5 μm thickness prepared on glass slides were deparaffinized in xylene and rehydrated via ethanol and placed in PBS. Antigen retrieval was performed by boiling the sections for 10 minutes in 0.01 M citrate buffer (pH 6.0) in a microwave oven. All sections were pretreated with a 3% aqueous solution of H_2O_2 for 5 minutes to quench endogenous peroxidase. Sections were then treated with 3% goat serum for 1 hour at room temperature to reduce nonspecific staining followed by 1 hour incubation with a human monoclonal anti-VEGF receptor-1 antibody, which recognizes the extracellular domain of the human VEGF receptor-1 (present in the sFlt-1). The antibody was used at a concentration of 10 μg/ml. The remainder of staining procedure was performed using a Universal Mouse Kit (Biogenex, Sam Ramon, CA), which contained biotinylated goat linking antibody to mouse immunoglobulins and an HRP–streptavidin complex. Diaminobenzidine tetrahydrochloride (DAB) was used as a substrate for the visualization of antigen–antibody complex. Slides were minimally counterstained with hematoxylin. To determine the degree of neovasculature, tumor tissues from naïve and rAAV-sFlt-1-treated mice, harvested at the same time, were processed as above. Detection of blood vessel was performed with a rat anti-mouse CD31 monoclonal antibody at a concentration of 10 μg/ml. The number and area of blood vessels in each group were counted in at least 10 different areas in a double-blinded method.

In situ *hybridization*

A digoxigenin (DIG)-labeled DNA probe containing the CMV promoter sequence was generated by PCR using the PCR-DIG labeling mix^{Plus} (Roche Molecular Diagnostics) following the manufacturer's protocol. Formalin-fixed tissues were sectioned to 5 μm thickness, deparaffinized in xylene and rehydrated through a series of graded-ethanol and PBS. Slides were then treated with 0.01 M citrate buffer, pH 6.0 at 42°C for 3 hours. Prehybridization was performed at 65°C for 2 hours in hybridization solution (ULTRAhyb^{TM}, Ambion, TX). The hybridization reaction was carried out with approximately 400 ng/ml of the DIG-labeled DNA probe at 65°C overnight. After thoroughly washing the excess probe, immunohistochemical detection of hybridization signals was performed using the DIG nucleic acid detection kit (Roche Molecular Diagnostics, Indianapolis, IN). Counterstaining of the slides was carried out with a diluted eosin solution for 1–2 minutes and then mounted with Crystal/Mount (Biomeda, Forest City, CA).

Statistical analyses

The association of the presence or absence of tumor with differing treatment conditions was tested for statistical significance by using the χ^2-test. The distribution of the tumor volume was measured against a normal (Poisson's distribution) following log transformation. In a general linear mixed model of the data, the dependent variable was the logged tumor volume and the predictors were treatment, day after treatment and "an interaction event" between treatment and day 0. All three were subsequently shown to be statistically significant predictors. The values of blood vessel area from naïve and rAAV-sFlt-1 were compared using the Student's t-test. P-values $< .05$ were considered to indicate significant difference between data sets.

Results

Generation of high-titer rAAV encoding human sFlt-1 for skeletal muscle injection

Previous studies have established sustained expression of rAAV transgenes in skeletal muscle without diminution of

expression or deterioration of transgene-expressing cells by cytolytic T cells. Hence, in the present study, we have chosen skeletal muscle as a platform for rAAV administration and production of sFlt-1 as a secretory protein. The human sFlt-1 cDNA was isolated from a HUVEC cDNA library[17] and subcloned in an rAAV vector under the control of the human cytomegalovirus immediate early promoter (CMV) (Fig 1). The rAAV was packaged in a helper virus-free system and purified using discontinuous iodixanol gradient centrifugation and affinity chromatography. The titer of the vector ranged between 1×10^{12} and 5×10^{12} particles/ml.

Determination of sFlt-1 expression as a secretory protein

The rAAV-sFlt-1 was tested in 293 cells for the extracellular secretion of the factor. Since the cDNA of cloned sFlt-1 contained the native VEGF receptor secretory signal, no additional modifications were performed to achieve extracellular transport of sFlt-1 from the transduced cells. The cells were either mock-transduced or transduced with 100 MOI of the rAAV encoding sFlt-1. The conditioned medium, obtained 48 hours after transduction, was concentrated five-fold and separated on SDS-PAGE and Western blot analysis performed using a monoclonal antibody, which recognizes human sFlt-1. Results, shown in Figure 2, clearly indicated a high-level expression of human sFlt-1 as a secretory protein following rAAV transduction in 293 cells. The antibody, recognizing sFlt-1 in supernatants obtained only from the rAAV-transduced 293 cells but not from the mock-transduced cells also indicated the specificity of detection.

Determination of biological activity of sFlt-1 following rAAV transduction

In the next set of experiments, we determined the biological activity of rAAV-expressed sFlt-1. We based our therapeutic approach on the well-established property of rAAV transgene expression as a secretory protein in skeletal muscle. Thus, the *in vitro* evaluation of the biological activity was performed mimicking the *in vivo* strategy. The rAAV-containing sFlt-1 gene was transduced into 293 cells and the transgene product was obtained as a secreted protein in the supernatant. The supernatant was added to early passage HUVEC, grown in the presence of 10 ng/ml VEGF. After 72 hours, the cells were fixed and stained with crystal violet. The proliferation index was determined by microphotography. Results, given in Figure 3, indicated a significant inhibition of HUVEC proliferation following the addition of supernatant from rAAV-sFlt1 transduced cells but not from the mock-transduced cells. As a positive control, purified recombinant human angiostatin was used at a concentration of 20 μg/ml, which also showed a significant inhibition of HUVEC proliferation. Highest inhibition, comparable to that from purified angiostatin, was observed with 25 μl supernatant indicating that an amount of approximately 125 ng/ml is produced in the supernatant of rAAV-sFlt-1-transduced cells.

Figure 1 Recombinant AAV encoding human sFlt-1. rAAV-containing human sFlt1 was subcloned under the control of the CMV promoter. Poly A represents the SV40 late polyadenylation signal sequence and ITR represents the inverted terminal repeat sequence of AAV.

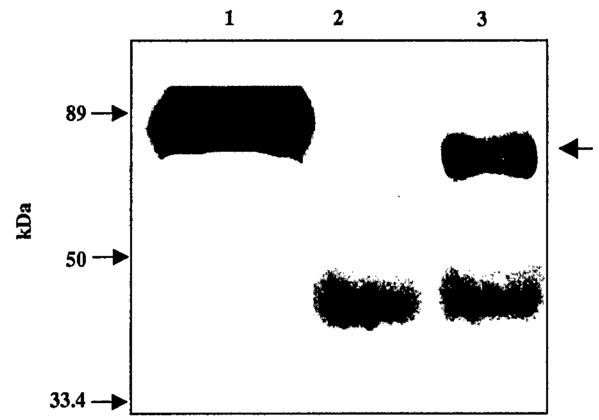

Figure 2 Western blot analysis of rAAV-sFlt-1 expression. 293 cells were either mock-transduced (2) or transduced with rAAV encoding sFlt-1 (3). At 48 hours after transduction, the supernatant was analyzed by Western blotting using a monoclonal antibody against human sFlt-1. A recombinant purified sFlt-1 protein, fused to the Fc-portion of immunoglobulin was used as a positive control (lane 1). A band of approximately 50 kDa, from nonspecific hybridization is seen in both mock-transduced and rAAV-sFlt-1-transduced lanes.

Development of a xenograft tumor model and vector treatment

As a model system to evaluate the *in vivo* antiangiogenic effects of rAAV-mediated long-term expression of sFlt-1, we developed subcutaneous tumors with an angiogenesis-dependent human ovarian cancer cell line SKOV3.ip1. Our pilot studies indicated that palpable tumors begin to appear 8–10 days following subcutaneous implantation of 10^7 SKOV3.ip1 cells in nude mice (data not shown). Since we based the present studies to evaluate the potential of AAV-mediated sFlt-1 gene therapy as a possible preventive therapy against the growth and recurrence of solid tumors, the animals were first injected with rAAV encoding sFlt-1. Also because optimal expression of rAAV transgenes is not achieved until at least 2 weeks after vector administration, tumor challenge was performed 3 weeks after vector administration. Each animal received an injection in both flanks. All the naïve animals and rAAV-GFP administered animals developed palpable tumors by 8 days after injection. The animals that developed tumors were monitored until the tumor volume reached 1800 mm^3 and euthanized according to the IACUC approved guidelines. Tumor-free animals were monitored for 130 days before terminating the experiment.

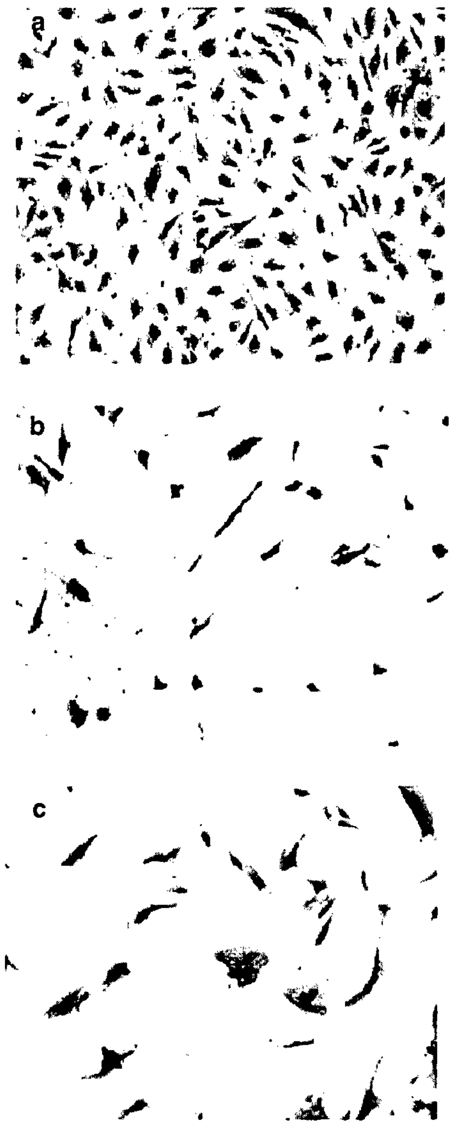

Figure 3 Endothelial cell proliferation assay to determine the biological activity of rAAV-sFlt-1. rAAV encoding sFlt-1 was transduced into 293 cells at an MOI of 100. After 48 hours, 25 μl of supernatant from mock-transduced (**a**) or rAAV-sFlt-1transduced (**b**) cells was tested on HUVEC. Cell proliferation was determined by fixing and staining the HUVEC with crystal violet. Purified recombinant human angiostatin was used at a concentration of 20 μg/ml (**c**) as a positive control. Magnification $\times$ 20.

Evidence of transgene persistence and expression following rAAV-sFlt-1 therapy in vivo

A major advantage of using rAAV in muscle-based gene therapy is the stable retention of transgene(s) for long-term expression. Since antiangiogenic gene therapy is directed towards inhibiting proliferation of tumor neovasculature and not tumor cells directly, an important requirement for antiangiogenic therapy is stable expression of these factors at therapeutic levels. Studies have reported that administration of rAAV in muscle not only results in long-term expression of the transgene but also that vector administered in this way does not elicit any host immune response against the transgenic protein, which would otherwise eliminate transgene-expressing cells.[4,24] Thus, in the present study, we sought to achieve this result by using skeletal muscle as a target tissue for transduction of rAAV and systemic secretion of sFlt-1. To demonstrate the long-term persistence of the AAV transgene, we performed genomic PCR and *in situ* hybridization, and to determine the systemic secretion of sFlt-1 from transduced muscle, we performed immunohistochemistry.

PCR analysis of vector genome was performed using total DNA isolated from naïve or rAAV-sFlt-1 treated mouse muscle. The forward and reverse primers consisted sequences of CMV promoter and human sFlt-1 coding sequences respectively. An amplification product of 548 bp, only in rAAV-sFlt-1 treated mice (Fig 4(i)) confirmed the persistence of vector genome. For *in situ* hybridization analysis, we used a digoxigenin (DIG)-labeled CMV promoter sequence as the probe. Results, shown in Figure 4(iii), also demonstrate the persistence of the AAV transgene in the skeletal muscle of only the vector-treated mice after 130 days. The use of CMV-promoter sequence as a probe in the *in situ* hybridization experiment also eliminated the possibility of hybridization signal from transgenic mRNA transcripts as well as cellular Flt-1 genomic DNA. Although the results of PCR or *in* situ hybridization do not confirm the integration of the rAAV genome into the host, increasing evidence indicate that only a small amount of rAAV transgenes integrate *in vivo*.[25] Nonetheless, in skeletal muscle, concatemerization of the rAAV genome allows long-term retention of transgenes as circular episomes.[6,7]

Immunohistochemistry was performed in paraffin sections of the transduced muscle and tumor tissues from naïve and rAAV-sFlt-1-treated mice to determine the expression of human sFlt-1 and degree of neovasculature respectively. Data, shown in Figure 4b, indicates systemic secretion of human sFlt-1 from the quadriceps muscle, only from rAAV-sFlt1-treated mice. Since the present study was performed as a preventive approach, tumor growth was not observed in disease-free animals in the sFlt-1 group. However, when the tumor sections were stained with CD31 antibody, there was a significant decrease ($P<.04$) in the blood vessel area in rAAV-sFlt-1 group suggesting the inhibitory effect on blood vessel growth.

Stable expression of sFlt-1 inhibits tumor growth in vivo

The results of the *in vivo* studies on growth kinetics of SKOV3.ip1 cells and tumor-free survival with a vector dose of 3×10^{11} particles are given in Figures 5 and 6, respectively. However, when a vector dose of 3×10^{10} particles was used, although there was a modest increase in tumor-free survival and a lesser mean tumor volume was noted, these effects were not statistically significant (data not shown). Palpable tumors started to develop 8 days after tumor challenge in naïve animals. When a dose

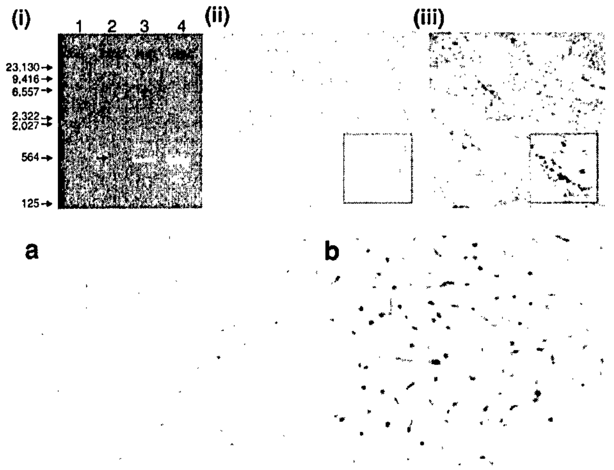

Figure 4 PCR, *In situ* hybridization and immunohistochemistry for long-term transgene persistence and expression. Top panel: PCR was performed using genomic DNA isolated from naïve or rAAV-sFlt-1-injected muscle using a primer-pair specific for the amplification of the transgene. Whereas no amplification product was seen in DNA from naïve mice (lanes 1 and 2), a 548 bp fragment, specific for the transgene, was amplified from rAAV-sFlt-1-treated mice (lanes 3 and 4, (i)). Sham- or rAAV-transduced muscle tissues were harvested and immediately fixed in buffered-formalin for 24 hours and then embedded in paraffin. *In situ* hybridization was performed using a DIG-labeled DNA probe on sections obtained from naïve (ii), or rAAV-sFlt1 (iii) administered muscle tissues. A two-fold magnification of a specific area is indicated in the box, showing positive signal only in the vector-transduced group. Bottom panel: Immunohistochemistry was performed using a human VEGF receptor-1 antibody, which recognizes the extracellular domain of VEGF receptor. Extracellular secretion of the transgenic factor from the vector-transduced muscle tissue is evident in rAAV-sFlt1 treated animal (**b**) compared to naïve mouse (**a**).

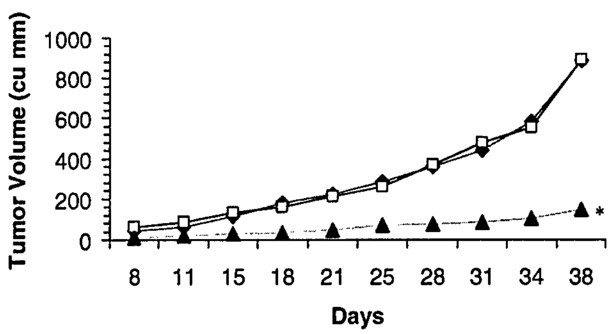

Figure 5 Growth characteristics of subcutaneously implanted SKOV3.ip1 cells in athymic nude mice following therapy with rAAV-sFlt-1. Three weeks after sham (◆), 3×10^{11} AAV-GFP (□), or rAAV-sFlt1 (▲) injection, 10^7 SKOV3.ip1 cells were subcutaneously implanted. Tumor size was measured using a digital caliper. The average tumor volume in each group is given in mm^3. *indicates P value <.0001, compared to naïve and AAV-GFP groups.

of 3×10^{10} particles of rAAV-sFlt1 was given, tumor-free survival was seen in >33% of the animals (P>.05) whereas a dose of 3×10^{11} particles protected 83% of the mice (P<.005). At a higher vector dose, there was also a significant difference in the mean tumor volume between

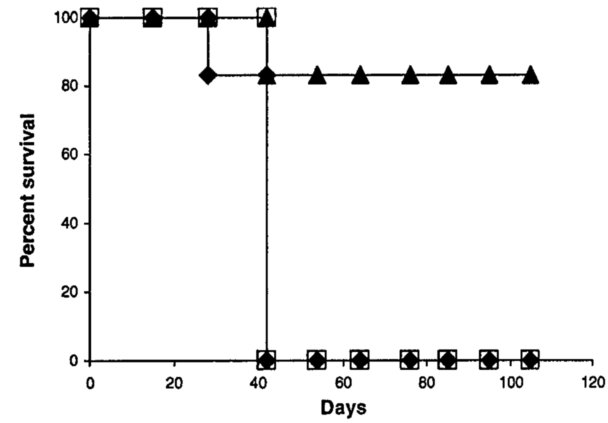

Figure 6 Tumor-free survival of mice following rAAV-sFlt-1 therapy. A total of 3×10^{11} particles of rAAV encoding GFP, or sFlt1 were injected into the quadriceps muscle in the hindlimb of athymic nude mice. At 3 weeks after the vector injection, the animals were challenged with 10^7 SKOV3.ip1 cells in each flank subcutaneously and tumor-free survival recorded. The mean tumor-free survival of naïve (◆), rAAV-GFP (□), or rAAV-sFlt1 (▲) is given above.

different treatments (naïve or GFP versus sFlt-1, P<.0001). There was a slight delay in the appearance of tumors in the rAAV-GFP-treated group compared to naïve animals, which did not receive any vector.

rAAV-sFlt-1 therapy does not cause liver damage

Previous studies have indicated that higher levels of sFlt-1 in mouse liver cause significant hepatotoxicity associated with hemorrhage of blood vessels in the liver.[14] VEGF has been reported to regulate the proliferation and survival of the sinusoidal endothelial cells acting through the VEGF receptors Flt-1 and Flk-1.[26,27] Blockade of the receptors, resulting from higher concentrations of sFlt-1 leads to such damage. Thus, overexpression of sFlt-1 from systemically delivered adenovirus vector, which targets liver, has been reported to result in similar damage.[14] To determine if stable systemic expression of sFlt-1 by rAAV leads to liver toxicity, liver tissues were macroscopically and microscopically examined following rAAV-sFlt-1 treatment. H&E-stained sections from naïve and AAV-sFlt-1-treated mice showed no cytomorphological signs of hepatotoxicity strongly suggesting that systemic expression of sFlt-1 from skeletal muscle does not result in liver toxicity (Fig 7). Further, immunohistochemical staining for human sFlt-1 in the liver sections did not reveal accumulation of systemically expressed sFlt-1 (data not shown) indicating that delivery of rAAV-sFlt-1 in skeletal muscle and systemic secretion of the factor at stable levels would be advantageous. Also, several studies using rAAV encoding a variety of therapeutic proteins have shown stable systemic expression of transgenic factors following intramuscular vector administration. No apparent effects such as loss of body weight, general mobility or food uptake was observed in the rAAV-sFlt-1-treated mice compared to untreated mice that were not tumor challenged.

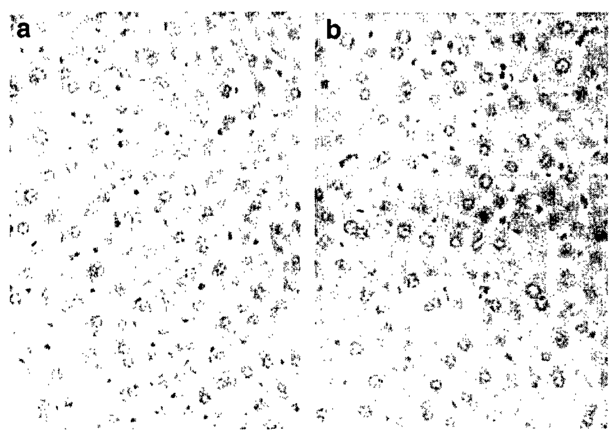

Figure 7 Absence of hepatotoxicity following rAAV-sFlt-1 therapy. Livers from naïve or rAAV-sFlt-1-treated mice were formalin-fixed, sectioned to 5 μm thickness and stained with H&E. The stained slides were analyzed by light microscopy for hepatotoxicity including hemorrhage and congestion, focal necrosis with minimal inflammation, hepatocyte dropout, or increased apoptosis. There was no difference that existed in the histopathology of liver sections between naïve mice (**a**) and rAAV-sFlt1-treated mice (**b**).

Discussion

Recent studies have indicated the potential of sFlt-1 gene therapy in murine models of human diseases using viral vectors.[15,28–32] Whereas studies using adenoviral and retroviral vectors have reported the efficacy of sFlt-1 gene therapy in inhibiting the growth of human tumors, that using AAV reported the effects in retinal diseases and *in vivo* growth of a stably transduced cell line.[31,32] The present study demonstrates that stable expression of sFlt-1 as a secretory protein from skeletal muscle by rAAV provides significant protection and long-term survival of mice against the growth of a human tumor xenograft suggesting its potential in cancer gene therapy.

The potential use of rAAV-sFlt1 as a vector for antiangiogenic therapy of solid tumors is multifold. Since tumor angiogenesis is mediated by a cascade of signals provided by cells of both tumor and endothelial origin, which ultimately results in the growth and differentiation of endothelial cells forming the tumor neovasculature, therapy targeting such an event should be sustained to effect a maximal tumoristatic response. Stable expression of transgenic factors as secretory proteins by administering rAAV in skeletal muscle has been well established in preclinical models[6,7,33–37] and formed the basis for a human clinical trial.[38] Thus, the proven efficiency of muscle-based administration of rAAV-sFlt-1 should provide sustained antiangiogenic effects. Since sFlt-1 is a native protein, there is no expected host immune response against the transgenic factor when used for therapy in humans. Further, the cytolytic T-cell response against rAAV-transduced muscle is known to be minimal due to both low-immunogenicity against the vector and poor transduction of rAAV to dendritic cells.[24] Thus, the potential of muscle-based rAAV-sFlt-1 therapy should be advantageous. Although several drugs have shown promise in controlling tumor neovasculature, a major problem in pharmacotherapy is the profound side effects of constant administration due to their limited half-life.[39] Gene therapy, on the other hand, offers advantages of maximizing cost effectiveness while maintaining sustained levels of antiangiogenic factors.

Although many factors are known to play important roles in new blood vessel formation, a key molecule promoting the growth of tumor neovasculature is VEGF, which has been considered a predictive marker in many human cancers.[9–13] Further, overexpression of VEGF mRNA and elevated serum VEGF levels have been correlated with decreased survival in many neoplastic conditions including ovarian cancer.[40] Despite higher levels of plasma VEGF levels observed in many human cancers, there was no concomitant increase in sFlt-1 levels in cancer patients indicating an imbalance in the native antiangiogenic signal pathway.[41] In patients who showed earlier relapse of breast cancer, tumor VEGF levels were higher than in patients with a longer disease-free survival and the rate of response to chemotherapy decreased with higher VEGF levels.[42] Hence, a stable gene therapy approach targeting VEGF as an adjuvant therapy offers the promise of increased survival in patients.

Inhibition of endogenous VEGF levels by the administration of VEGF antibody alone or in combination of topoisomerase inhibitors has resulted in effective immunotherapy and reduction of Wilms' tumor respectively in murine models.[43,44] Unlike other biologically driven antiangiogenic factors such as angiostatin and endostatin whose mechanism of action are not fully elucidated despite their antiangiogenic effects, the biological properties of sFlt-1 are well known. The sFlt-1 acts by both sequestering VEGF and blocking VEGF receptors from binding to VEGF.[8] Thus, therapy targeting VEGF will have specific effects on tumor growth inhibition and metastasis.

We have recently reported that intravenous administration of adenovirus encoding sFlt-1 results in systemic toxicity due to high sFlt-1 expression in liver.[14] By using an adenovirus encoding GFP, the toxicity was determined to be due to increased sFlt-1 levels rather than vector localization in liver. Thus, using skeletal muscle as a platform to achieve systemically therapeutic levels of sFlt-1, rAAV can overcome such a deleterious effect in addition to offering the advantage of long-term expression. In a recent preclinical study, we also demonstrated that significant inhibition of tumor growth can be achieved by intramuscular administration of rAAV encoding angiostatin and endostatin as secretory proteins.[45] Histopathological analysis of liver from rAAV-sFlt-1 treated mice liver also confirmed the absence of any cytomorphic damage.

Results of our studies additionally indicated that significant therapeutic effect was seen only with a vector dose of 3×10^{11} particles in mice. Thus, a clinical translation of these findings would require careful determination of optimal vector dose. Although we did

not quantitatively determine the systemic levels of sFlt-1 following therapy, at the therapeutically efficacious dose, immunohistochemical analysis of rAAV-sFlt-1 injected muscle and adjacent blood vessels clearly indicated abundant expression and secretion of sFlt-1, suggesting that delivery of the sFlt-1 transgene in muscle is a safer method of overcoming any potential toxicity due to systemic delivery of a recombinant adenovirus encoding sFlt-1.

A recent study on the potential use of rAAV encoding sFlt-1 in ovarian cancer reported that transduction of the human ovarian cancer cell line, RMG-1, with AAV-sFlt1 *in vitro* followed by intraperitoneal administration in nude mice resulted in a decrease in proliferative and metastatic indices, further suggesting the feasibility of localized AAV-sFlt-1 antiangiogenic gene therapy.[32] However, a major limitation of intratumoral delivery is the limited transduction efficiency and dispersion of the vector within the tumor cells. Also, unlike certain genetic metabolic diseases, which require only partial amounts of the deficient protein/enzyme for phenotypic correction of the disease, tumor therapy requires inhibition of the tumor growth *in toto*. Antiangiogenic therapy, in particular, requires a constant level of the inhibitory factor(s) for a sustained therapeutic effect. Thus, the strategy that we adapted in the present study will likely have greater potential and translational utility for human cancers, in particular, as an adjuvant therapy against tumor recurrence. Although several preclinical studies have shown that stable levels of systemically secreted proteins using rAAV resulted in the phenotypic correction of inherited metabolic defects,[6,7] accumulation of antiangiogenic factors in other organs due to unregulated expression may lead to ischemic conditions or impair wound healing. Thus, future studies are warranted to test the efficacy of regulated expression of these factors by using inducible promoters[46,47] for a safe muscle-based rAAV antiangiogenic gene therapy.

Acknowledgments

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Recombinant Adeno-Associated Virus 2-Mediated Antiangiogenic Prevention in a Mouse Model of Intraperitoneal Ovarian Cancer

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ABSTRACT

Purpose: **In the present study, we sought to determine the potential of sustained transgene expression by a single i.m. administration of recombinant adeno-associated virus 2 (rAAV) encoding angiostatin and endostatin in inhibiting i.p. ovarian cancer growth and dissemination in a preclinical mouse model.**

Experimental Design: **Cohorts of female athymic nude mice received either no virus or 1.2 × 10^{11} particles of rAAV encoding green fluorescence protein or endostatin plus angiostatin, i.m. Three weeks later, the mice were i.p. injected with 10^6 human epithelial ovarian cancer cell line SKOV3.ip1. As a measure of effectiveness of the therapy, tumor weight, abdominal distension, ascites volume and vascular endothelial growth factor level, and tumor weight were determined. Immunohistochemistry was done to determine tumor cell apoptosis and endothelial cell proliferation following the therapy. Tumor-free survival was recorded as the end point.**

Results: **Results indicated a significant tumor-free survival ($P < 0.003$) following therapy with rAAV encoding endostatin and angiostatin compared with untreated or rAAV-green fluorescence protein–treated mice. Ascites volume in rAAV endostatin and angiostatin–treated mice was significantly lower than naive mice and contained less hemorrhage and tumor conglomerates. The level of vascular endothelial growth factor in the ascites of antiangiogenic vector treated mice was also significantly less compared with the untreated mice. Immunohistochemical analyses indicated increased tumor cell apoptosis and decreased blood vasculature following rAAV endostatin and angiostatin treatment.**

Conclusion: **The results indicate that antiangiogenic genetic prevention from stable systemic levels of angiostatin and endostatin by i.m. administration of rAAV can be used for the treatment of i.p. ovarian cancer growth and dissemination.**

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INTRODUCTION

Ovarian cancer is the second most common gynecologic malignancies in women (1). Because ovarian carcinoma frequently remains clinically silent, a majority of patients with the disease have advanced i.p. dissemination during diagnosis. The mean survival rate for disseminated ovarian cancer is <5 years (2, 3). Despite a better understanding of the disease pathology, surgery, and chemotherapy remain the major therapeutic interventions for ovarian cancer. Like most of the solid tumors, ovarian cancer growth and metastasis is dependent on new blood vessel formation by the process of angiogenesis (4, 5). Thus, therapies targeting angiogenesis are promising for the control of tumor growth in patients with ovarian cancer. Because the tumoristatic antiangiogenic therapy targets endothelial cells, effects of this therapy should be sustained without toxicity. Gene therapy approaches seem promising for this purpose.

We recently showed using a recombinant adeno-associated virus (rAAV) that stable systemic expression of antiangiogenic factors following i.m. vector administration results in significant inhibition of a human epithelial ovarian cancer cell line, SKOV3.ip1, grown as a s.c. xenograft in nude mice (6). Although this study showed the effects of rAAV antiangiogenic gene therapy, unlike s.c. tumors, the growth of epithelial ovarian cancer is highly disseminative, accompanied by excessive i.p. ascites and exfoliation of tumor cells in the peritoneal cavity, which limits the efficacy of drugs and other therapeutic molecules from reaching tumor cells. The presence of excess ascites at the time of laparotomy for ovarian cancer patients has also been associated with poor prognosis (7) and the amount of vascular endothelial growth factor (VEGF) in ascites correlates to the disease pathology (8, 9).

Thus, in the present study, we sought to determine if rAAV-mediated expression of angiostatin and endostatin as secretory factors following i.m. administration of the vector will reduce VEGF levels, ascites burden, and prolong survival in a preclinical mouse model of i.p. ovarian cancer. The results indicate the effectiveness of this molecular therapy.

MATERIALS AND METHODS

Cells and Reagents. Human embryonic kidney cell line 293 was purchased from American Type Culture Collection (Manassas, VA) and maintained in Iscove's modified essential medium supplemented with 10% newborn calf serum. The human epithelial ovarian cancer cell line SKOV3.ip1 was a gift from Dr. Janet Price (M.D. Anderson Cancer Center, Houston, TX) and was maintained in Eagle's MEM containing nonessential amino acids and 1 mmol/L sodium pyruvate supplemented with 10% fetal bovine serum. The cells were cultured in 37°C with 5% CO_2. The SKOV3.ip1 cells were harvested from

subconfluent culture by brief exposure to 0.25% trypsin, stopped with medium, containing 10% serum, were washed twice, and resuspended in PBS. Only single-cell suspension with >95% viability was used for *in vivo* injection.

Restriction endonucelases and other modifying enzymes were purchased from either New England Biolabs (Beverly, MA) or Promega Co. (Madison, WI). A mouse monoclonal antibody for Ki67 (clone SP6) and a rabbit polyclonal poly(ADP-ribose) polymerase (PARP) p85 fragment were obtained from Research Diagnostics, Inc. (Flanders, NJ) and Promega, respectively. Secondary antibodies and color reagents were purchased from Amersham (Piscataway, NJ). The mouse VEGF ELISA kit was purchased from R&D System, Inc. (Minneapolis, MN).

Recombinant Plasmids, Production, and Purification of rAAV. Construction of recombinant plasmids containing secretable form of human angiostatin and endostatin as bicistronically expressed proteins and that encoding green fluorescence protein (GFP) was recently published (6). Production and purification of rAAV was done by transient transfection in 293 cells followed by iodixanol gradient centrifugation and heparin affinity column chromatography (10). The particle titer of purified virions was determined by quantitative slot blot analysis (11).

***In vivo* Studies.** Six-week-old female athymic nude mice were purchased from the National Cancer Institute-Frederick Animal Production Area (Frederick, MD). All the animal studies were done in accordance with guidelines of the Institutional Animal Care and Use Committee, and all experimental procedures were approved by the Institutional Animal Care and Use Committee and the Occupational Health and Safety Department of the University of Alabama at Birmingham. Approximately 10^{11} genomic particles of rAAV encoding GFP or endostatin and angiostatin, in normal saline, were injected in a volume of 100 μL in the quadriceps muscle in the hind limbs. Naive animals did not receive any vector. Each group consisted of 10 mice. Three weeks after vector administration, each mouse received 10^6 SKOV3.ip1 cells by i.p. injection. The onset of i.p. tumors was determined based on significantly increased abdominal circumference due to ascites. Comparisons were made between animals in the control (mice without AAV or tumor challenge), naive (mice with SKOV3.ip1 cells but no rAAV), and rAAV (rAAV GFP/ endostatin + angiostatin + SKOV3.ip1 challenge) groups for abdominal volume. The abdominal area was carefully checked to detect palpable tumors in these groups. The animals were monitored twice every week for body weight and tumor formation in the peritoneal cavity and were euthanized when they become moribund, the day of euthanasia considered as the limit of survival. Ascites fluid and peritoneal tissues were harvested for further analyses. The weight of solid peritoneal tumors and ascites volume were recorded. Blood samples were collected from all animals before vector administration, before tumor cell implantation, and at sacrifice.

Immunohistochemistry. Immunohistochemical studies were done in 5-μm sections of paraffin-embedded tumor tissues using antibodies for Ki67 and anti-PARP p85 for the determination of proliferation and apoptosis indices respectively. Antigen retrieval was achieved by incubating the slides in 0.05% trypsin for 20 minutes at 37°C and endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 10 minutes in room temperature. The anti-Ki67 monoclonal antibody was used in a working dilution of 1:50, and the anti-PARP p85 polyclonal antibody in a dilution of 1:50. Furthermore, the slides were stained with a donkey anti-rabbit horseradish peroxidase–linked secondary antibody (1:500 dilution). To determine the proliferation and apoptotic indices, stained slides were examined under high power (×40). A minimum of 10 randomly chosen fields were counted to determine the total number of cells and that stained positive in each field. The percentage proliferation and apoptosis was calculated using the formula: (number of positively stained cells/total number of cells in a field) × 100. The antigen-antibody complex was visualized with diaminobenzidine tetrahydrochloride, and tissues were counterstained minimally with hematoxylin.

ELISA. Ascites fluid was harvested on the day of sacrifice of the animals due to tumor burden and the volume measured. The ascites fluid was briefly centrifuged to remove loose cells and the supernatant frozen at −80°C until analysis. The VEGF levels in ascites were determined using a commercial ELISA kit (R&D System), which recognized the 164 and 120 amino acid residues of mouse VEGF.

Toxicity Analysis. Hepatic toxicity was assessed by quantitative measurement of serum alanine aminotransferase using a commercial kit (TECO Diagnostics, Anaheim, CA), and histopathology of serial liver sections by H&E staining. The effects of rAAV treatment in other major peritoneal organs, including kidney, ovary, and spleen were also determined histopathologically by H&E staining of tissues obtained from control and rAAV-treated mice.

Statistical Analysis. Data were compiled as mean ± SE in quantitative experiments. For statistical analysis of differences between the groups, an unpaired Student's t test was done. $Ps < 0.05$ were considered to indicate significant difference between data sets.

RESULTS

Development of Mouse Model of i.p. Ovarian Cancer. An experimental model of ovarian cancer was developed with 6-week-old female athymic nude mice. To establish i.p. tumor, each mouse was injected with 10^6 SKOV3 i.p.1 cells i.p. Mice that developed ascites or lost 10% of body weight were euthanized. With this amount of tumor cells, mice developed palpable tumor between days 15 and 20. Upon sacrifice, in the peritoneum, visible tumors were found under the diaphragm, intestine, and in the peritoneal cavity. The mean survival of untreated mice was found to be 45 days after tumor cell implantation.

Treatment with rAAV Endostatin and Angiostatin Significantly Decreases Ascites Volume and VEGF Levels. Because SKOV3.ip1 cells form tumors around 2 weeks following i.p. administration and that optimal expression of rAAV transgenes does not occur until 2 to 3 weeks after vector injection, the present study was designed to pretreat the mice with rAAV before tumor challenge. Based on the kinetics of rAAV transgene expression, we evaluated the therapeutic

Fig. 1 Recombinant AAV encoding endostatin and angiostatin as bicistronically expressed proteins. rAAV containing human endostatin and angiostatin as a bicistronic cassette was subcloned under the control of the CMV promoter. *PolyA*, SV40 late polyadenylation signal sequence; *ITR*, inverted terminal repeat sequence of AAV.

efficacy of a one-time i.m. injection of rAAV endostatin and angiostatin (Fig. 1) before tumor cell implantation. Cohorts of mice were given 10^{11} genomic particles of rAAV encoding endostatin and angiostatin as bicistronically expressed proteins from a single vector or rAAV encoding GFP to determine vector-related nonspecific effects.

By serum ELISA, we determined that this dose of vector produced a mean 196 ng/mL endostatin and 227 ng/mL angiostatin systemically beginning 3 weeks. The peak level expression achieved at 3 weeks after vector injection was found to be stable for over 4 months without any diminution or toxicity. The SKOV3.ip1 cells were implanted i.p. at a dose of 10^6 cells per mouse. Approximately 2 weeks following tumor cell injection, abdominal distension was noted in the group of mice, which received no treatment. However, in the group of mice treated with rAAV endostatin and angiostatin, there was a significant decrease in the ascites volume ($P < 0.05$) as shown in Fig. 2. In addition, the ascites from naive mice was more hemorrhagic, whereas that from rAAV endostatin and angiostatin treated group was clear. The VEGF levels in ascites fluid also showed a significant decrease in the group of mice treated with rAAV endostatin and angiostatin ($P < 0.05$) indicating the specificity of the antiangiogenic effect (Fig. 3).

Recombinant AAV Endostatin Plus Angiostatin Therapy Decreases Tumor Cell Proliferation and Increases Apoptosis. The mean tumor weight in rAAV endostatin and angiostatin–treated group was significantly less ($P < 0.0002$) compared with naive mice as shown in Fig. 4. To determine if stable systemic levels of endostatin and angiostatin from rAAV transgene effects on the proliferation and apoptosis of i.p. ovarian cancer cells, immunohistochemistry was done with Ki67 and anti-PARP antibodies respectively. The *in situ* effects of angiostatin and endostatin gene transfer are illustrated in immunohistochemical analysis of tumor cell proliferation and apoptosis compared with naive animals (Fig. 5). Whereas >30% of implanted tumor cells in the rAAV-treated group were apoptotic compared with animals in the naive group, no significant differences was found between the naive and rAAV-GFP–treated animals ($P > 0.05$, data not shown) indicating the specificity of the transgenic factors. The median proliferation index of tumor cells in rAAV endostatin

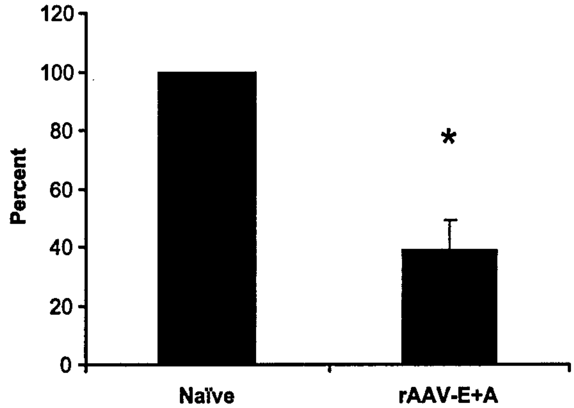

Fig. 3 Ascites VEGF levels following rAAV endostatin + angiostatin therapy in ascites volume. VEGF level in the ascites was determined by ELISA. *Columns*, mean compared with that obtained in naive mice as 100%; *bar*, ±SE. *, $P < 0.0001$ compared with naive mice.

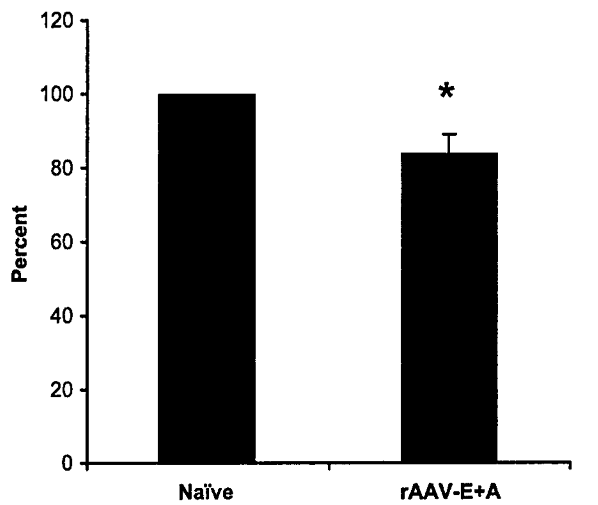

Fig. 2 Effects of rAAV endostatin + angiostatin therapy in ascites volume. The mean ascites volume was determined in naive and rAAV endostatin + angiostatin–treated mice following peritoneal lavage and expressed as percentage relative to levels obtained in naive mice. *, $P < 0.05$ compared with naive animals.

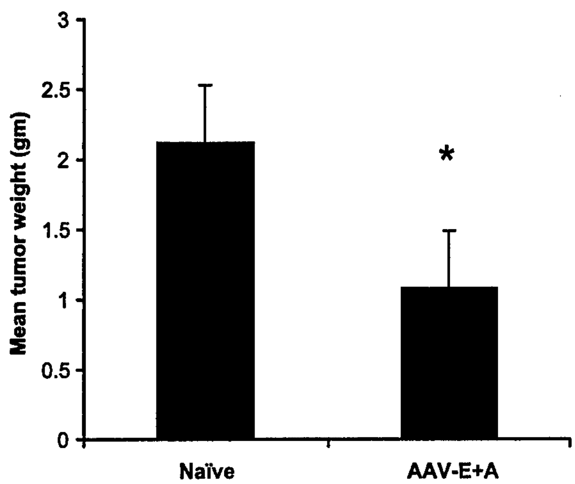

Fig. 4 Effects of rAAV endostatin + angiostatin gene therapy in tumor burden. Tumor nodules from the peritoneal cavity of naive and rAAV endostatin + angiostatin–treated mice were collected and weighed. *Columns*, mean weight of tumors. *, $P < 0.0002$ compared with naive animals.

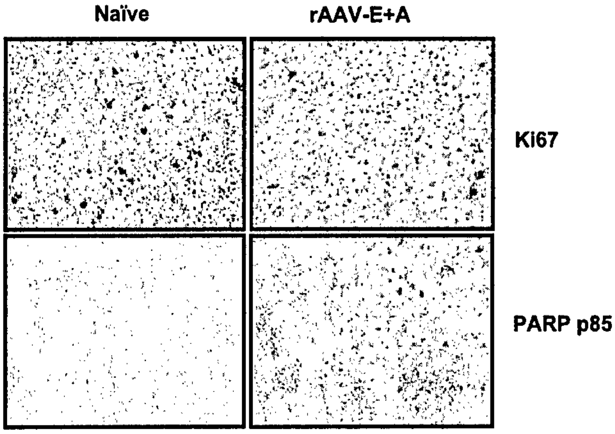

Fig. 5 Immunohistochemical analysis of tumor tissues for tumor cell proliferation and apoptosis. I.p. tumor nodules were isolated during sacrifice and fixed in buffered formaldehyde. Immunohistochemical staining to determine tumor proliferation and apoptosis was done in 5-μm sections using the Ki67 and anti-PARP antibody, respectively, and visualized under a light microscope. Magnification × 20.

and angiostatin treated mice was 22%, significantly less than in control group, which showed a proliferation index of 61%.

Stable Antiangiogenic Gene Transfer Increases Tumor-free Survival of Mice. Following tumor cell implantation, mice were monitored at least twice every week as described in Materials and Methods for tumor growth up to 150 days when the experiments were terminated. The result of tumor-free survival is shown in Fig. 6. There was a significant protective effect of AAV-mediated antiangiogenic gene expression with both endostatin and angiostatin compared with control or rAAV-GFP–treated animals ($P < 0.003$). There were no apparent effects such as loss of body weight, general mobility, or food uptake in the long-term surviving rAAV- endostatin and angiostatin–treated mice compared with untreated mice that were not tumor challenged. Serum alanine aminotransferase levels, as a measure of hepatotoxicity, suggested no difference between control and rAAV treatment groups (Fig. 7). The absence of microscopic abnormalities in liver, ovary, spleen, and kidney in rAAV-treated group was also confirmed by histopathology (data not shown).

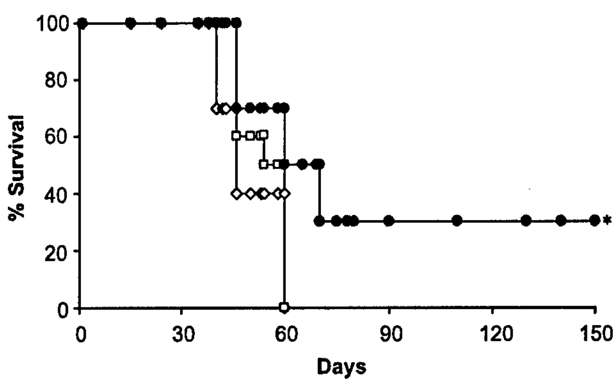

Fig. 6 Tumor-free survival following rAAV therapy. Following no treatment (◇) or i.m. injection of 10^{11} particle of rAAV encoding GFP (□) or endostatin + angiostatin (•), 10^6 SKOV3.ip1 cells were i.p. injected. The animals were monitored for body weight, abdominal distension, and peritoneal tumor growth. When the animals became moribund or the tumor size/abdominal volume reached Institutional Animal Care and Use Committee stipulated limits, they were euthanized. Tumor-free animals were maintained for 150 days before terminating the experiment. *, $P < 0.003$ compared with naive and rAAV-GFP–treated animals.

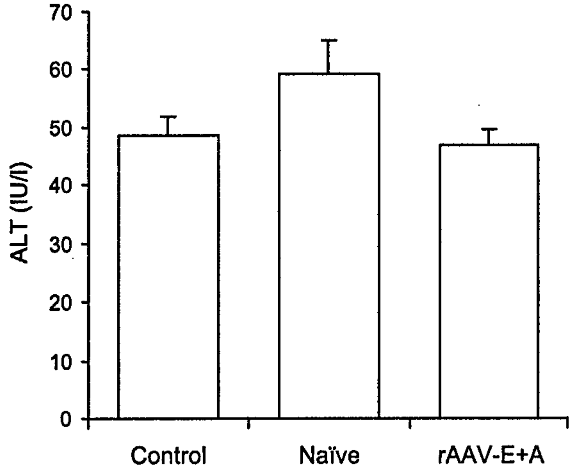

Fig. 7 Serum alanine aminotransferase (*ALT*) measurement following rAAV therapy. Serum samples from control (untreated and unchallenged), naive (no treatment but challenged with SKOV3.ip1 cells), and rAAV (endostatin + angiostatin and challenged with SKOV3.ip1) were used to determine the ALT levels by a colorimetric method. *Points,* mean ± SD.

DISCUSSION

Although the growth of ovarian cancer depends on neovasculature, unlike organ-localized solid tumors, the growth of i.p. ovarian cancer is highly disseminative. The end-stage disease is characterized by the accumulation of i.p. ascites fluid, decreasing intracellular levels of given drugs, posing a great challenge. We recently showed that production of systemically stable levels of angiostatin and endostatin by rAAV gene transfer abrogated the growth of s.c. implanted human ovarian cancer cell line SKOV3.ip1 (6). As a logical extension of these studies, we determined the effects of stable systemic expression of angiostatin and endostatin following a single i.m. administration of the vector on i.p. growth and dissemination of SKOV3.ip1 cells.

Results indicated a significant protective effect of rAAV-mediated expression of endostatin and angiostatin against i.p. growth of ovarian cancer in nude mice. Peritoneal dissemination of ovarian cancer originates by their release into ascites, which initiates the process of metastasis. Many reports have suggested that the major angiogenic factor which plays an important role in the vascularization of neoplastic tissue and ascites formation is VEGF, also known as vascular permeability factor. The VEGF induces ascites accumulation by increasing the permeability of diaphragmatic and tumor-associated vasculature (12, 13).

Previous studies have reported that administration of monoclonal antibody to human VEGF can prevent ascites formation in a mouse model of i.p. ovarian carcinoma with SKOV3 cells (14). Compared with protein or pharmacotherapies, gene transfer approach provides greater benefit of stable

systemic levels of the antiangiogenic factors. The advantages of using rAAV over other vectors are nonpathogenicity, long-term transgene expression, and absence of vector-related cellular immune response (15, 16). Our studies established that a single i.m. administration of rAAV encoding angiostatin and endostatin results in systemic levels of these factors between 177 to 277 and 176 to 206 ng/mL, respectively, in serum after 3 weeks and remained stable for over 4 months without any apparent toxicity.

The antiangiogenic mechanism of endostatin and angiostatin are beginning to be discovered. Although angiostatin seems to exert antiangiogenic effect by primarily inhibiting the proliferation and invasion of endothelial cells (17, 18) and inducing endothelial cell apoptosis (19), endostatin reduces endothelial cell proliferation (20) and migration (21) and significantly reduces the invasion of endothelial as well as tumor cells into the reconstituted basement membrane (22). Thus, a combination of angiostatin and endostatin is likely to increase the inhibitory effect on ascites formation as observed in the present studies.

The results of these studies indicate that antiangiogenic gene therapy by stable systemic levels of angiostatin and endostatin following i.m. administration of rAAV can be used for the treatment of i.p. ovarian cancer growth and dissemination. This strategy may be combined with other therapies, including chemotherapy to increase the survival of ovarian cancer patients. A recent study involving i.m. administration of rAAV encoding angiostatin in human glioma xenografts (23) and i.m. administration of rAAV endostatin in a colorectal cancer model have also shown therapeutic efficacy (24). However, the growth characteristics of i.p. ovarian cancer limit intratumoral administration due to dissemination of the tumor cells and poor transduction efficiency of the primary tumors. Thus, administration of the vector in skeletal muscle may result in a therapeutically stable systemic level of the factors. Due to the lack of an effective screening method, insidious onset and nonspecific symptoms, a majority of women present with late-stage disease. In this situation, cytoreductive surgery and chemotherapy form the major therapeutic interventions. Although these therapies have increased the initial clinical response rates, recurrent disease remains a formidable challenge. As in the primary disease, the growth of recurring ovarian cancer is highly dependent on angiogenesis. Hence, antiangiogenic prevention of recurring cancer may prove beneficial in extending survival rates. Furthermore, ~ 10% to 15% of the patients develop the disease due to genetic predisposition. To such "at-risk" population, this approach could possibly help to delay or prevent the onset of the primary cancer.

Although in our studies we did not observe any toxicity following rAAV therapy, if constant systemic levels of the antiangiogenic factors prove toxic to patients, the approach presented in this study may be improved to achieve localized production of the factors within the tumor. Furthermore, although several preclinical studies have shown that stable levels of systemically secreted proteins using rAAV resulted in the phenotypic correction of inherited metabolic defects (25), accumulation of antiangiogenic factors in other organs due to unregulated expression may lead to ischemic conditions or impair wound healing. Thus, future studies are warranted to test the efficacy of regulated expression of these factors by using inducible promoters, for a safe muscle-based rAAV antiangiogenic gene therapy.

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